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# The *Arabidopsis thaliana rlp* mutations revert the ectopic leaf blade formation conferred by activation tagging of the *LEP* gene

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**Abstract** Activation tagging of the gene *LEAFY PETIOLE* (*LEP*) with a T-DNA construct induces ectopic leaf blade formation in Arabidopsis, which results in a leafy petiole phenotype. In addition, the number of rosette leaves produced prior to the onset of bolting is reduced, and the rate of leaf initiation is retarded by the activation tagged *LEP* gene. The ectopic leaf blade results from an invasion of the petiole region by the wild-type leaf blade. In order to isolate mutants that are specifically disturbed in the outgrowth of the leaf blade, second site mutagenesis was performed using ethane methanesulphonate (EMS) on a transgenic line that harbours the activation-tagged *LEP* gene and exhibits the leafy petiole phenotype. A collection of *revertant for leafy petiole* ( *rlp*) lines was isolated that form petiolated rosette leaves in the presence of the activated *LEP* gene, and could be classified into three groups. The class III *rlp* lines also display altered leaf development in a wild-type (non-transgenic) background, and are probably mutated in genes that affect shoot or leaf development. The *rlp* lines of classes I and II, which represent the majority of revertants, do not affect leaf blade outgrowth in a wild-type (non-transgenic) background. This indicates that LEP regulates a subset of the genes involved in the process of leaf blade outgrowth, and that genetic and/or functional redundancy in this process compensates for the loss of RLP function during the formation of the wild-type leaf blade. More detailed genetic and morphological analyses were performed on a selection of the *rlp* lines. Of these, the dominant *rlp* lines display complete reversion of (1) the leafy petiole phenotype, (2) the reduction in the number of rosette leaves and (3) the slower leaf initiation rate caused by the activation-tagged *LEP* gene. Therefore, these lines are potentially mutated in genes for interacting partners of LEP or in downstream regulatory genes. In contrast, the recessive *rlp* lines exhibit a specific reversion of the leafy petiole phenotype. Thus, these lines are most probably mutated in genes specific for the outgrowth of the leaf blade. Further functional analysis of the *rlp* mutations will contribute to the dissection of the complex pathways underlying leaf blade outgrowth.

**Keywords** Arabidopsis leaf mutants · Leaf blade outgrowth · *LEP* · *rlp* · Suppressor mutants

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## Introduction

Given the important role of leaves in photosynthesis, respiration and photoreception, the proper development of the leaf blade is essential for the successful growth and reproduction of plants. The systematic isolation and subsequent analysis of leaf-shape mutants in Arabidopsis has resulted in the description of many mutants affected in a wide range of aspects of leaf blade formation (Berna et al. 1999; Serrano-Cartagena et al. 1999). However, only a few Arabidopsis mutants form bladeless leaves. The *phb* and *phv* mutants (McConnell and Barton 1998; McConnell et al. 2001) are disturbed in the process of dorsalisation and produce radially symmetrical leaves, which resemble the leaves produced by the *phan* mutant in snapdragon (Waites and Hudson 1995). Dorsalisation of the developing leaf primordium has been shown to be the key step preceding leaf blade formation. The resulting juxtaposition of dorsal and ventral tissues serves as a trigger for subsequent cell divisions at the margins of the leaf primordium, which in turn lead to leaf blade outgrowth (Waites and Hudson 1995; Poethig 1997; Waites et al. 1998; Van Lijsebettens

and Clarke 1998). Other genes that are involved in the establishment of dorsoventrality include members of the *YABBY* family, which encode transcription factors, while the *KANADI* genes specify ventral cell fate, and the *PINHEAD/ZWILLE* and *AGO1* genes specify dorsal cell fate. The functional analysis of these genes has shown that genetic redundancy is a feature of the process of dorsalisation in all leaf-like organs, including floral organs (Lynn et al. 1999; Bowman 2000a, 2000b; Byrne et al. 2001; Eshed et al. 2001; Kerstetter et al. 2001).

The outgrowth of the leaf blade is a complex process involving both cell division and cell elongation (Donnelly et al. 1999). Hitherto, the *lam-1* mutant in *Nicotiana sylvestris* (McHale 1992, 1993) is the only dicotyledonous leaf mutant in which the leaf blade initiation site is established normally following dorsalisation of the leaf primordium. However, the subsequent cell divisions that would normally result in leaf blade outgrowth are disturbed (McHale and Marcotrigiano 1998), so that the leaves in this mutant consist only of the midrib. The Arabidopsis *add3* mutant (Pickett et al. 1996) shows a defect in the formation of the leaf blade that partly resembles the *lam-1* phenotype. However, the exact defect in the *add3* mutant still needs to be resolved.

Recently, we reported that activation tagging, with a T-DNA construct, of the gene for the AP2/EREBP like transcription factor LEP results in the dominant *lettuce* (*let*) mutation, which induces the formation of ectopic leaf blades and gives rise to a leafy petiole phenotype (van der Graaff et al. 2000). In wild-type plants *LEP* is expressed in leaf primordia and young leaf blades. In the *let* mutant the activation of *LEP* by the transgenic tag has led to cell- and tissue-specific upregulation of *LEP* expression. Transgenic plants harbouring the activation tagged *LEP* gene also exhibit a leafy petiole phenotype, similar to that displayed by the *let* mutant (van der Graaff et al. 2000). The ectopic leaf blade phenotype conferred by the activation of the tagged *LEP* gene is symmetrical along the proximodistal axis. Furthermore, all cell layers, and the vascular patterning, of the ectopic leaf blade, are continuous with those of the wild-type leaf blade. Therefore, the activation tagged *LEP* gene causes the wild-type leaf blade to extend further downwards, so that it invades the petiole region, most probably because of increased LEP activity in the petiole region. In contrast, constitutive and ectopic overexpression of *LEP* results in increased and ectopic cell divisions in all aerial organs, suggesting that in wild-type plants LEP controls cell division during leaf blade outgrowth.

It has proven to be difficult to isolate Arabidopsis mutants that are specifically disturbed in the outgrowth of the leaf blade following dorsalisation of the leaf primordium. This might result from genetic and/or functional redundancy (Franco-Zorrilla et al. 2002), which would be expected to prevent the isolation of loss-of-function mutants for this process. The fact that a *lep* insertional mutant does not display a loss-of-function phenotype (van der Graaff et al. 2002) strengthens this hypothesis. This in turn implies that the process of cell division during leaf blade outgrowth is regulated by more than one gene. These regulators could either control the expression of the same set of genes involved in leaf blade outgrowth, or each could control a distinct subset. If the activation tagged *LEP* gene affects the expression of only a subset of the genes involved in leaf blade outgrowth, it might offer a tool for the isolation of mutants that are specifically disturbed in this process. Consequently, mutagenesis of any of these genes in an activated *LEP* background should cause a loss of the ectopic leaf blade phenotype. Because of the proposed functional/genetic redundancy in the process of leaf blade outgrowth, the loss-of-function in such a mutant should be compensated during wild-type leaf blade development and, consequently, the leafy petiole phenotype should revert to wild type. If each of the regulators controls the same set of genes, a mutation in one of their target genes should not affect the development of either wild-type or ectopic leaf blades.

Here, we report the isolation of *revertant for leafy petiole* (*rlp*) mutant lines following EMS mutagenesis of a transgenic line that harbours the activation tagged *LEP* gene and exhibits the leafy-petiole phenotype. These *rlp* lines form petiolated leaves in the presence of the activated *LEP* gene. We present a genetic and morphological description of the *rlp* lines, using leaf dimensions, leaf initiation rate and numbers of rosette leaves produced prior to the onset of bolting as parameters to quantify the strength of the reverting mutations.

## Materials and methods

### Plant material

Plants were grown as described previously (van der Graaff et al. 2000). Ecotype C24 was used as the wild-type control for the analysis of leaf dimensions, leaf number and leaf initiation rate for *let*, the parental line 30 W and the *rlp* lines. Lehle Seeds (Round Rock, Tex.) performed EMS mutagenesis on 0.6 g of seeds from the hemizygous 35SDE- *LEP* transgenic line 30 W. The T-DNA insert in this parental line confers BASTA resistance, so selection for the T-DNA was applied by adding the herbicide to the irrigation water throughout the EMS mutagenesis procedure. M2 seeds were obtained from twelve independent pools of 1000 M1 seeds each. In all, 48,000 M2 seeds were screened for mutants that exhibited an altered leaf shape compared to the parental line. Briefly, 3000 seeds (Screen 1) and 1000 seeds (Screen 2) per M2 family were grown in soil, and BASTA selection was applied by watering and spraying. Putative revertants (which formed petioles on rosette leaves 5–8) were confirmed in the M3 and M4 generation. In order to analyse the nature of the revertant mutations, BASTA-resistant siblings exhibiting a revertant leaf phenotype in the M3 generation were backcrossed to wild-type C24 plants. The BC-F2 progenies obtained from BASTA resistant BC-F1 plants were then analysed for the segregation of the revertant mutations.

### Determination of leaf size and leaf initiation rate

Plants were grown in soil (5 plants per 5 cm pot), or in tissue culture (50 plants per 14.5 cm petri dish) on half strength MS medium (Murashige and Skoog 1962)]. One week after the onset of bolting all rosette leaves were removed, and the leaf dimensions were measured using a dissection microscope equipped with a

millimetre scale. The leaf initiation rate was determined by counting the emerging leaves daily using a dissection microscope.

### Molecular analysis

Isolation of total RNA and Northern hybridisation analysis were performed as described previously (van der Graaff et al. 2000). PCR analysis was performed with primers specific for the 35S promoter: 35S-1 (5′-GCTCCTACAAATGCCATCA-3′) and 35S-2 (5′-GATAGTGGGATTGTGCGTCA-3′).

### Mapping of the *rlp* mutations

Pollen from BC-F2 BASTA-resistant revertant siblings was used to fertilise wild-type L*er* plants. F2 progenies were then obtained from the BASTA resistant F1 plants generated by these crosses, and those that segregated for the revertant leaf phenotype and the T-DNA insert (BASTA resistance) were selected for mapping analysis. The mapping populations were grown under BASTA selection (50 plants per 14.5 cm petri dishes). For each *rlp* line, 24 revertant siblings were selected after 3 weeks of growth, and DNA was isolated using a rapid method (Liu et al. 1996). For mapping analysis of the additional mutations in the lines *rlp* 3-8 (small shoots) and *rlp* 12-14 (organ fusion), seeds were grown on petri dishes without BASTA selection from those F2 populations that segregated for these additional mutations. Standard SSLP and CAPS markers (Konieczny and Ausubel 1993; Bell and Ecker 1994; Lukowitz et al. 2000) and the uzu markers (C. Ringli, unpublished results) were used to determine the chromosomal position of each of the *rlp* mutations.

## Results

### Isolation of the *rlp* lines

In order to identify genes that are specifically involved in the outgrowth of the leaf blade, we performed a revertant screen based on the leafy petiole phenotype conferred by the activation tagged *LEP* gene. The line 30 W, which carries the transgene 35SDE-*LEP* (van der Graaff et al. 2000) was used as the parental line for second site mutagenesis by EMS. In this line the *LEP* transgene is strongly expressed, and the plants are characterised by a strong leafy petiole phenotype, similar to that of *let* (Figs. 1B, C and 2B, C). The parental line, like *let*, forms fewer rosette leaves before the onset of bolting (Fig. 1C) and leaf initiation occurs at a slower rate than in wild type (Fig. 1D).

Following EMS mutagenesis of the parental line 30 W, plants were selected that exhibited petiolated leaves in the transgenic background. Reversion of the leafy petiole phenotype in such mutagenised transgenic plants can be caused by several different mechanisms. Mutations in the 35S CaMV promoter that drives expression of the *LEP* transgene can result in a decrease in, or complete loss of, transgenic *LEP* expression. Otherwise, mutations in the *LEP* transgene can either alter LEP activity or result in loss-of-function. In the case of such intragenic mutations, the revertant mutation will be genetically linked to the parental T-DNA insert. Extragenic mutations resulting in a loss of the ectopic leaf blade formation can be caused by mutations in genes for putative interacting partners of LEP or genes involved in leaf blade formation that are regulated by LEP.

In total, 48,000 EMS-mutagenised M2 seeds were screened (under selection for BASTA resistance to select for the T-DNA; see Materials and methods) for leaf shapes that deviated from the leafy petiole phenotype displayed by the parental line (Fig. 2C). This resulted in the isolation of 65 putative mutants, of which the majority showed the formation of petiolated leaves for rosette leaves 5–8 in the presence of the transgene and, therefore, were putative *revertant for leafy petiole* (*rlp*) mutants. A minority of the 65 lines displayed a more severe leafy petiole phenotype. However, these enhancer mutations were infertile and therefore could not be analysed further.

Seeds were obtained from 53 of the putative *rlp* lines. For 38 of these, a clear reversion of the leafy petiole phenotype was confirmed in the M3 generation

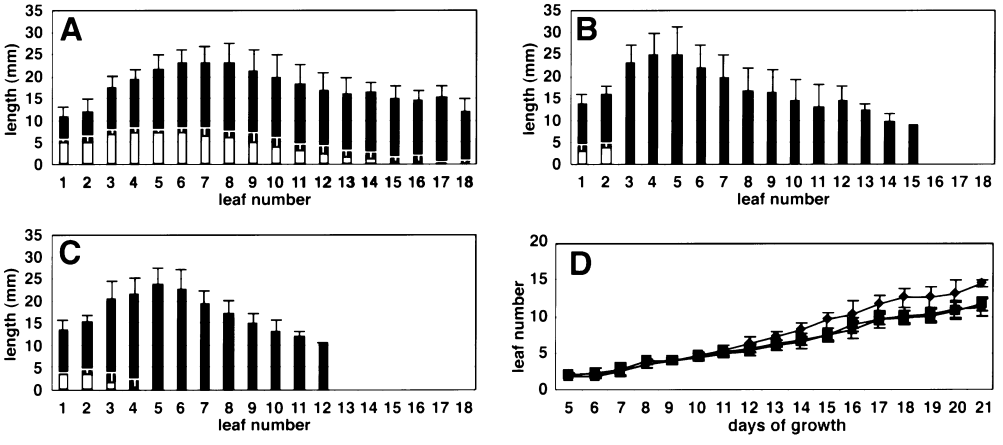


**Fig. 1A–D** Activation tagging of *LEP* causes ectopic leaf blade formation, reduces the number of rosette leaves produced before the onset of bolting, and results in a lower rate of leaf initiation. The histograms in panels **A**, **B** and **C** depict leaf dimensions and leaf numbers for wild type (n = 29), **B** *let* (n = 15) and the parental line (30 W; n = 10), respectively. The *open bars* indicate petiole length (± SD); the *filled bars*, leaf blade length (± SD). **D** Leaf initiation rate for wild type ( *diamonds*, n = 50), *let* ( *squares*, n = 17) and the parental line (30 W; *triangles*, n = 21). n is the number of plants analysed

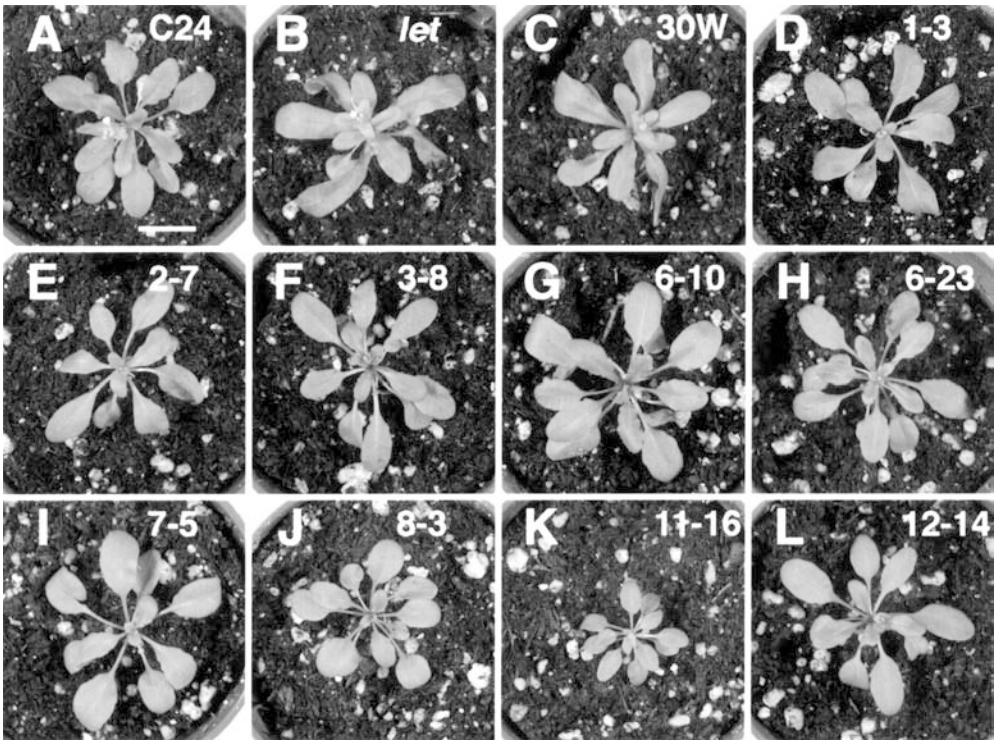


**Fig. 2A–L** The leafy petiole phenotype is conferred by the activation tagged *LEP* gene and is reversed by the *rlp* mutations. The plants shown were grown for 25 days in soil. **A** Wild-type (C24). **B** *let*. **C** Parental line (30 W). **D–F** Recessive *rlp* lines 1-3, 2-7 and 3-8, respectively. **G–J** Dominant *rlp* lines 6-10, 6-23, 7-5 and 8-3, respectively. **K** Recessive class III line *rlp* 11-16. **L** Recessive line *rlp* 12-14. *Scale bar* 1 cm

(Table 1). The 28 class I *rlp* lines display a strong and specific reversion of the leafy petiole phenotype, while the class II *rlp* lines show a weak reversion. The 10 class III *rlp* lines show a strong reversion of the leafy petiole phenotype, but also exhibit additional shoot phenotypes. In the majority of the class III lines this additional shoot phenotype consists of the formation of pale green (and smaller) shoots or pointed leaves. All the progeny of the class III lines with a revertant leaf phenotype display an additional shoot phenotype, whereas their siblings with the (parental) leafy petiole phenotype are unaffected. Therefore, the mutations that induce these additional shoot phenotypes are linked to the *rlp* mutations. The additional shoot phenotypes were also observed in the wild-type (non-transgenic) siblings of these lines. This indicates that the class III lines are most likely to be disrupted in genes that affect shoot formation, and thereby prevent the establishment of the leafy petiole phenotype. In contrast, the class I and II *rlp* lines do not affect leaf blade development in a wild-type (non-transgenic) background. Thus, only the class I and II *rlp* mutations are specific for the function of LEP. In some cases, several *rlp* lines that exhibited a similar revertant phenotype were isolated from one M2 family, suggesting that these lines were derived from the same parent.

**Table 1** Classification of the *rlp* lines

| Class[a] | Number of lines | Phenotype |
|---|---|---|
| I | 28 | Strong reversion of leafy petiole phenotype |
| II | 15 | Petiolated leaf, but reversion of leafy petiole phenotype is weak |
| III | 10 | Strong reversion, but additional (linked) shoot phenotype[b] |

[a]The 53 *rlp* lines with a confirmed revertant leaf phenotype in the M3 generation were grouped into three classes based on their respective revertant leaf and shoot phenotypes

[b]All revertant siblings also display an additional shoot phenotype. Therefore, this additional phenotype is linked to the revertant mutation

## Genetic and morphological analysis of the *rlp* lines

The integrity of the T-DNA in the *rlp* lines was studied by PCR analysis using primers specific for the 35S promoter that activates the expression of the *LEP* transgene. These primers detected the T-DNA insert carried by the parental line in all 53 *rlp* lines (data not shown) with a confirmed revertant phenotype in the M3 generation. Furthermore, the expression levels of the *LEP* transgene in these *rlp* lines were similar to that in the parental line, except in the case of *rlp* 6-23 (Fig. 3). These results exclude the possibility that the revertant leaf phenotype in the *rlp* lines is caused by (intragenic) mutations that affect the expression levels of the *LEP* transgene.

The nature of the *rlp* mutations was analysed based on their segregation in the progenies obtained from backcrosses (BC) of the *rlp* lines to wild-type plants (see Materials and methods). In the case of intragenic mutations or dominant mutations that are genetically tightly linked to the T-DNA insert harboring the *LEP* transgene, all BASTA resistant BC-F1 and BC-F2

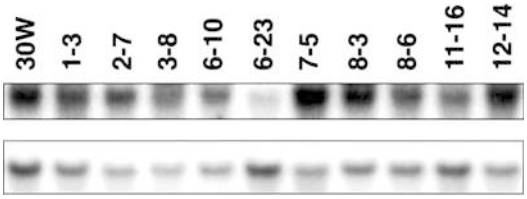


**Fig. 3** The expression level of the *LEP* transgene is unaffected in all but one of the *rlp* mutants. RNA was isolated from 3-week-old shoots. At this stage no expression of the wild-type *LEP* gene can be detected. The Northern blot (20 μg total RNA) was hybridised with the *LEP* coding region amplified from cDNA (*upper panel*), stripped and then hybridised with the *GapC* coding region amplified from cDNA (*lower panel*) to control for loading

siblings should display a revertant phenotype. This was observed for five of the *rlp* lines (Table 2). Dominant *rlp* mutations that are genetically unlinked to the T-DNA insert would result in a 1:3 segregation for leafy petiole:revertant siblings under BASTA selection (selection for the parental T-DNA) and 3:13 without such selection. Recessive revertant mutations that are genetically unlinked to the T-DNA insert would segregate 3:1 for leafy petiole:revertant siblings under BASTA selection and 9:7 without selection. In the absence of selection for the parental T-DNA insert, both the revertant siblings and the wild-type (non-transgenic) siblings were classified as revertant. For most of the *rlp* lines, the *rlp* mutations segregate independently of the T-DNA insert that includes the activated *LEP* transgene, and the majority of these *rlp* mutations clearly segregate either as a recessive or as a dominant trait (Table 2).

Several *rlp* lines were selected for more detailed analysis, based on the strength of the reversion of the leafy petiole phenotype and the initial segregation analysis of their respective *rlp* mutations. Because the reversion of the leafy petiole phenotype is most prominent in the class I *rlp* lines, we focused on this class of revertants. Four recessive class I *rlp* lines, four dominant class I *rlp* lines and one class III *rlp* line (Fig. 2) were analysed in more detail. This analysis was performed on the BC-F2 progenies and, therefore, the *rlp* mutations and the parental T-DNA insert segregated independently in these progenies, which necessitated a more detailed segregation analysis of these *rlp* mutations (Table 3). In addition, the developmental defects caused by the *LEP* transgene (Fig. 1) were used to quantify the strength of the revertant mutations. The leaf dimensions (Fig. 4), the number of rosette leaves produced before the onset of bolting (Fig. 4) and the leaf initiation rate (Fig. 5) were analysed in the nine selected *rlp* lines and compared to corresponding values for the parental line (30 W) and the wild type.

The recessive class I lines *rlp* 1-3 (Figs. 2D and 4D), *rlp* 2-7 (Figs. 2E and 4F), *rlp* 3-8 (Figs. 2F and 2H) and *rlp* 12-14 (Figs. 2L and 4J) display a moderate reversion of the leafy petiole phenotype without affecting leaf width (data not shown), and their leaf initiation rate is comparable to that of the parental line (Fig. 5A). The lines *rlp* 3-8 and *rlp* 12-14 display an additional shoot phenotype that segregates independently of the *rlp* mutations and the parental T-DNA insert. Thus, this additional shoot phenotype in *rlp* 3-8 and *rlp* 12-14 is not related to the reversion of the leafy petiole phenotype, and these lines therefore do not belong to the class III *rlp* lines. The additional phenotype in *rlp* 3-8 consists of the formation of small shoots, and in *rlp* 12-14 leaves occasionally attach to other organs (data not shown), which resembles the phenotype displayed by transgenic Arabidopsis plants expressing a fungal cutinase (Sieber et al. 2000) and by the *ale1* mutant (Tanaka et al. 2001).

**Table 2** Genetic analysis of the 53 *rlp* lines

| Putative nature of the *rlp* mutations[a] | Number of lines |
|---|---|
| Recessive | 29 |
| Dominant | 11 |
| Genetically linked with 35SDE-*LEP*T-DNA insert[b] | 5 |
| Not determined[c] | 8 |

[a]The nature of the *rlp* mutations was analysed in the BC-F2 progenies, based on the segregation of the siblings displaying the parental phenotype (leafy petiole) versus the revertant leaf phenotype on medium BASTA to select for the presence of the T-DNA
[b]These mutations are either intragenic or dominant, and are genetically tightly linked to the 35SDE-*LEP* T-DNA insert
[c]Segregation ratios fit neither that of a dominant nor a recessive mutation

**Table 3** Genetic analysis of the selected *rlp* lines in the BC-F2 progenies

| *rlp* line | BASTA[a] | | Control[b] | | Nature of mutation[c] |
|---|---|---|---|---|---|
| | Leafy petiole | Revertant | Leafy petiole | Revertant | |
| Expected recessive ratio | 3 | 1 | 9 | 7 | Recessive |
| Expected dominant ratio | 1 | 3 | 3 | 13 | Dominant |
| 1-3 | 93 | 47 | 198 | 126 | Recessive |
| 2-7 | 105 | 35 | 194 | 104 | Recessive |
| 3-8[d] | 99 | 27 | 190 | 79 | Recessive |
| 6-10 | 31 | 110 | 69 | 193 | Dominant |
| 6-23 | 37 | 106 | 77 | 225 | Dominant |
| 7-5 | 36 | 100 | 48 | 159 | Dominant |
| 8-3 | 31 | 111 | 58 | 156 | Dominant |
| 11-16 | 102 | 35 | 164 | 90 | Recessive |
| 12-14[d] | 90 | 47 | 99 | 76 | Recessive |

[a]Segregation ratio on medium containing BASTA to select for the presence of the T-DNA
[b]Segregation ratio on medium without selection for the presence of the T-DNA. Both the revertant siblings and the wild-type (non-transgenic) siblings were classified as revertant
[c]The segregation data fit those expected for either a dominant or a recessive mutation
[d]These lines harbour an additional shoot mutation, unlinked to either the T-DNA insert or the *rlp* mutation. See text for further details

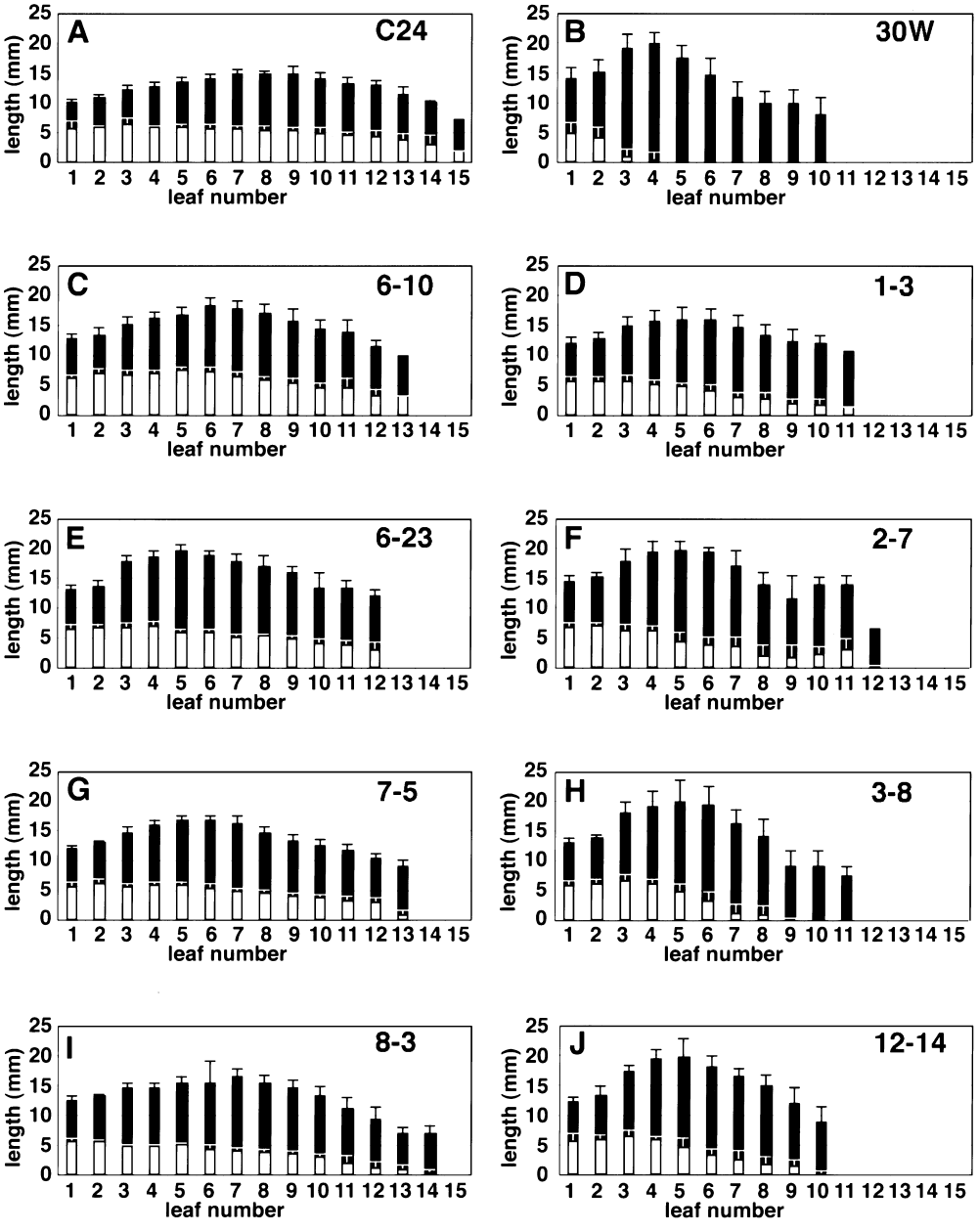


**Fig. 4A–J** Leaf dimensions and numbers of rosette leaves produced before the onset of bolting by selected *rlp* lines. **A** Wild type (n = 9). **B** Parental line (30 W; n = 10): **C**, **E** , **G** , **I** Dominant *rlp* lines 6-10 (n = 10), 6-23 (n = 10), 7-5 (n = 10) and 8-3 (n = 10), respectively. **D**, **F** , **H** , **J** Recessive *rlp* lines 1-3 (n = 10), 2-7 (n = 10), 3-8 (n = 10) and 12-14 (n = 6), respectively. The *open bars* indicate petiole length (± SD); the *filled bars*, leaf blade length (± SD). n is the number of plants analysed

In contrast to the recessive class I *rlp* lines, the recessive class III *rlp* 11-16 line exhibits a strong reversion of the leafy petiole phenotype (Fig. 2K) and a wild-type leaf initiation rate (Fig. 5A). All revertant *rlp* 11-16 siblings display the formation of small and pale shoots. Thus a comparison of the leaf dimension was not possible for *rlp* 11-16.

The dominant class I lines *rlp* 6-10 (Figs. 2G and 4C), *rlp* 6-23 (Figs. 2H and 4E), *rlp* 7-5 (Figs. 2I and 4G) and *rlp* 8-3 (Figs. 2J and 4I) exhibit a strong reversion of the leafy petiole phenotype without affecting leaf width (data not shown), and display a wild-type leaf initiation rate (Fig. 5A). For *rlp* 6-23 significantly lower expression of the *LEP* transgene was detected compared to the parental line (data not shown), which might be the reason for the complete reversion of the developmental alterations associated with the activation tagged *LEP* gene.

Since the *rlp* mutations and the parental T-DNA insert segregated independently in the BC-F2 progenies of the selected *rlp* lines, the development of the BASTA resistant siblings that exhibit the parental leafy petiole phenotype could also be studied. These siblings have the genotype *RLP* / *RLP* for the dominant lines and either *RLP* / *RLP* or *RLP* / *rlp* for the recessive lines. The number of leaves produced before the onset of bolting (data not shown), the leaf dimensions (data not shown) and the leaf initiation rate (Fig. 5B) for those siblings were similar to those of the parental line. This shows that the reversion of the leafy petiole phenotype and the other developmental alterations related to the activated *LEP* transgene are caused by the *rlp* mutations, and are

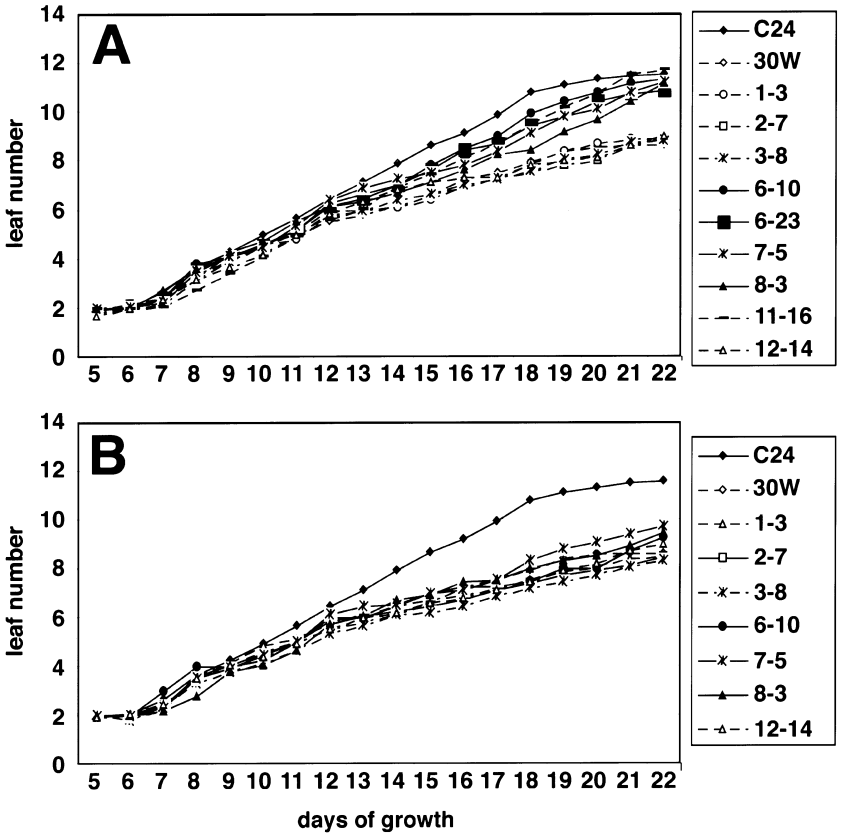


**Fig. 5A, B** Leaf initiation rate in selected *rlp* lines. Panel A shows the rates for the wild type (n = 41), the parental line (30 W; n = 19) and the siblings exhibiting a revertant leaf phenotype from the *rlp* lines 1-3 (n = 10), 2-7 (n = 12), 3-8 (n = 12), 6-10 (n = 32), 6-23 (n = 30), 7-5 (n = 12), 8-3 (n = 11), 11-16 (n = 38) and 12-14 (n = 6). Panel **B** shows the data for the siblings exhibiting the parental leafy petiole phenotype from the *rlp* lines 1-3 (n = 25), 2-7 (n = 29), 3-8 (n = 23), 6-10 (n = 4), 7-5 (n = 32), 8-3 (n = 29) and 12-14 (n = 26). The size of the error bars was comparable to those shown in Fig. 1. n is the number of plants analysed

not influenced by putative additional unlinked mutations affecting shoot growth in the *rlp* lines.

Crosses were carried out between the *rlp* lines and L*er* wild-type plants to generate mapping populations (see Materials and methods). Mapping analysis identified the map positions of the *rlp* mutations 1-3, 2-7, 3-8, 6-23, 7-5 and 12-14, whereas no obvious linkage with the available genetic markers was obtained for the *rlp* mutations 6-10, 8-3 and 11-16 (Table 4). Furthermore, the mutations that cause the additional shoot phenotypes in the class I *rlp* 3-8 and *rlp* 12-14 lines were mapped to different chromosomal locations from their respective *rlp* mutations, confirming that these additional shoot phenotypes are not related to the reversion of the leafy petiole phenotype in these *rlp* lines.

**Table 4** Mapping analysis of the mutations in the selected *rlp* lines

| *rlp* line | Marker | Chromosome | Region on chromosome |
|---|---|---|---|
| 1-3 | uzu7 | I | Bottom |
| 2-7 | Athbio2 | II | Bottom |
| 3-8 | nga162/uzu1 | III | Top |
| 6-23 | nga1126 | II | Middle |
| 7-5 | nga162/uzu1 | III | Top |
| 12-14 | nga162/uzu1 | III | Top |
| 3-8: small shoot[a] | uzu36 | IV | Bottom |
| 12-14: organ fusion[b] | uzu7 | I | Bottom |

[a]Additional unlinked mutation in the *rlp* line 3-8
[b]Additional unlinked mutation in the *rlp* line 12-14

## Discussion

Several Arabidopsis mutants have been described that affect the size of the leaf blade. The leaf size in the *ucu1* (Perez-Perez et al. 2002), *an* (Tsuge et al. 1996) and *rot* mutants (Tsuge et al. 1996) is altered because of a defect in cell elongation, while in *rev* (Talbert et al. 1995), *an3* (Tsukaya 2002) and *cro4* (Tsukaya 2002) leaf size is affected by an altered pattern of cell division. In the *clf* (Kim et al. 1998) and *ant* mutants (Mizukami and Fischer 2000) both cell division and elongation are perturbed, resulting in larger and smaller leaves, respectively. The activation tagging of the *LEP* gene in the *lettuce* mutant is the first example of a mutation that alters the ratio between leaf petiole and leaf blade length without affecting total leaf length or width. The mutant *asymmetric leaves* (Byrne et al. 2000; Semiarti et al. 2001) and the novel recessive *bop1* mutant (Ha et al. 2003) develop leaflet-like structures on leaf petioles. These structures result from newly initiated leaf blades rather than an invasion of the petiole region by the wild-type leaf blade such as that associated with activation tagging of the *LEP* gene. The *bop1* and *asymmetric leaves* mutants act via the control of class I *KNOX* gene expression, and double mutants between *bop1* and the *asymmetric leaves* exhibit more severe defects in leaf development than does either single mutant (Ha et al. 2003). In contrast, crosses between either *let* or an activation-tagged *LEP* transgene and the *asymmetric leaves* mutant only show additive effects (data not

shown). Thus, the leafy petiole phenotype arises by a mechanism that is distinct from those affected in published leaf blade mutants.

We have employed second site mutagenesis using EMS in a transgene background and screened for enhancers/suppressors of the leafy petiole phenotype conferred by the activation tagged *LEP* gene. Several enhancers of the leafy petiole phenotype were identified. However, these plants were infertile and could not be analysed further. The suppressor mutants display a reversion of the leafy petiole phenotype. Hence, we named these mutant lines *revertant for leafy petiole* (*rlp*).

## The class I and II *rlp* mutations are specific for the function of LEP

The majority of the *rlp* mutations segregated independently of the T-DNA insert in the parental transgenic line harbouring the activation tagged *LEP* gene. Furthermore, the T-DNA insert was intact in these lines and, except in the case of *rlp* 6-23, expression levels of the *LEP* transgene were unaffected. Therefore, the *rlp* mutations are genetically unlinked to the T-DNA insert. For eight of the *rlp* lines the segregation data for the *rlp* mutations did not comply with the expected ratio for either dominant or recessive mutations. These *rlp* mutations probably either result in a weak reversion of the leafy petiole phenotype, thereby hampering the segregation analysis, or the *rlp* mutations are located on the same chromosome as the T-DNA insert harboring the activation tagged *LEP* gene. In five *rlp* lines the *rlp* mutation appears to be genetically linked to the T-DNA insert. Plasmid rescue and subsequent sequence analysis of the *LEP* transgene should indicate whether intragenic mutations have occurred. Such mutants could reveal protein domains/motifs that are essential for the function of LEP.

All the siblings in the progeny of the class III *rlp* lines with a revertant leaf phenotype also display an additional shoot phenotype. Furthermore, these lines display aberrant leaf development in a wild-type (non-transgenic) background. Therefore, these class III *rlp* lines are most probably disrupted in genes that generally affect shoot or leaf formation, and thus fail to establish the leafy petiole phenotype. The class I and II *rlp* mutations neither exert pleiotropic effects on plant development nor affect leaf shape in a wild-type (non-transgenic) background. Furthermore, they suppress the leafy petiole phenotype without affecting total leaf length or width. Thus, these *rlp* mutations are specific for the function of LEP and, therefore, are most likely to represent novel mutations affecting leaf blade formation.

## The *rlp* mutations revert different aspects of plant development affected by the activation tagged *LEP* gene

Quantification of the revertant leaf phenotype displayed by the selected *rlp* lines showed that the dominant class I *rlp* lines exhibit the strongest reversion of the leafy petiole phenotype. Furthermore, the number of rosette leaves formed before the onset of bolting, and the leaf initiation rate, in the dominant *rlp* lines resemble the values for the wild type. Thus, the dominant *rlp* lines display a complete reversion of the alterations in plant development conferred by the activation tagged *LEP* gene. Mutations in regulatory genes like transcription factors and genes involved in signal transduction can often result in constitutively active or inactive proteins, thus inducing dominant phenotypes. Furthermore, such mutations can affect several aspects of development. Therefore, these dominant lines are possibly mutated in genes for interacting partners of LEP or in regulatory genes downstream of *LEP*. The mechanism underlying the revertant mutation in *rlp* 6-23 might, however, differ from that in the other dominant lines. Expression levels of the *LEP* transgene are strongly reduced in *rlp* 6-23. Because the *rlp* 6-23 mutation is genetically unlinked to the parental T-DNA it is not an intragenic mutation. Thus, the *rlp* 6-23 mutation affects the expression of the *LEP* transgene rather than LEP function. This could be caused by the mutation of a factor that binds to either the *LEP* promoter or to CaMV 35S promoter sequences. Since the CaMV 35S promoter depends on plant transcription factors for its activity, a mutation in any such factor might also result in altered expression of several plant genes. The *rlp* 6-23 mutation does not confer a visible phenotype in a wild-type (non-transgenic) background and, therefore, probably affects a factor that binds to the *LEP* promoter. A mutation that resulted in constitutive binding of a factor to the *LEP* promoter sequences might impair the expression of both the wild-type *LEP* gene and the *LEP* transgene, resulting in a dominant revertant phenotype.

Although the rosette leaves formed by the recessive *rlp* class I lines are clearly petiolated, the petiole length is still reduced compared to wild type. In contrast to the dominant class I *rlp* lines, the number of rosette leaves produced prior to the onset of bolting, and the leaf initiation rate, in the recessive *rlp* lines are comparable to those in the parental line. Because the mutations in the recessive class I *rlp* lines only affect the ectopic leaf blade and are recessive, these lines are most likely to be mutated in genes specific for the outgrowth of the leaf blade.

The class I and II *rlp* lines do not affect leaf blade outgrowth in a wild-type (non-transgenic) background. This indicates that LEP regulates a subset of the genes involved in the process of leaf blade outgrowth, and that other regulatory genes acting in parallel with LEP in leaf blade development control the expression of at least one other subset of genes involved in leaf blade development. Genetic and/or functional redundancy should exist between these different subsets of genes, because the loss of RLP function is compensated during the formation of the wild-type leaf blade in the *rlp* lines. Furthermore, little or no genetic and/or functional redundancy is present within the subset of genes regulated by LEP, since it was possible to isolate mutations that revert the

ectopic leaf blade phenotype conferred by the activation tagged *LEP* gene.

The difference between the dominant and recessive class I *rlp* mutations shows that the diverse developmental pathways affected by the activation tagging of *LEP* can be genetically separated. Recently, we reported that activation tagging of *LEP* also results in an increase in xylem cell numbers in the vascular tissue of all aerial organs (van der Graaff et al. 2002). In analogy to its role in leaf blade formation, LEP most probably controls cell division activity during xylem formation. Analysis of vascular tissue formation in the *rlp* mutants will indicate whether the vascular phenotype conferred by the activation tagged *LEP* gene is also reverted by the *rlp* mutations.

Interestingly, the dominant *bracts1-d* mutant, which was originally identified on the basis of ectopic bract formation (Dinneny et al. 2001), also displays a leafy petiole-like phenotype. Therefore, BRACTS could be a candidate regulator of cell-division activity during leaf blade outgrowth that functions in parallel to LEP. The *bracts1-d* mutant could therefore be used to analyse the specificity of the *rlp* mutations in reverting ectopic leaf blade formation.

Three of the *rlp* mutations were mapped to the same arm of chromosome III. More elaborate analysis is necessary to examine whether these *rlp* mutations represent independent loci. However, the *rlp* mutations only become manifest in the activation tagged *LEP* transgenic background, which hampers more detailed genetic analysis of the *rlp* lines. The identification of the genes mutated in the *rlp* lines offers a starting point for the elucidation of the complex genetic pathway(s) that underlie the process of leaf blade outgrowth, and will allow the dissection of the diverse pathways affected by the activation tagging of *LEP*.

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